# METABOLOMICS AND SYSTEMS BIOLOGY IN THE CONTEXT OF IN VITRO AND IN VIVO TOXICITY STUDIES: TOWARDS A BETTER UNDERSTANDING OF THE PROCESSES UNDERLYING THE DISRUPTION OF BIOLOGICAL SYSTEMS BY CHEMICAL POLLUTANTS

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## Introduction

The development of novel approaches allowing the modeling of the metabolic network of biological systems opens new perspectives for studies exploring the dangers associated with the presence of chemicals in the human food and environment. We previously demonstrated that the modulation produced by very low doses of EDC (Endocrine Disrupting Chemicals) could be detected when exposure occurs during the perinatal period[1]. However, in *vivo* studies are expensive, and there is a need to reduce the number of animals used for laboratory experiments. Thus, *in vivo* studies cannot systematically be carried out for all the chemicals listed in extensive screening programs, or which effects need to be investigated within the frame of specific regulations such as the EU REACH directive. It is expected that the use of human metabolically competent cell lines, combined with high resolution spectral analyses, will allow, in a first step, to identify "metabolic fingerprints" that will contribute to the screening of chemical pollutants. Then, based on a reconstruction of the metabolic network, and on comparative studies of closely related xenobitics (in terms of structure and/or effects), evidence for metabolic networks modulation should allow highlight major disruption routes of cellular metabolism. The use of non targeted approaches also opens the way to a better understanding of the mechanisms of toxicity of low doses of exposure, and of the effect of mixtures. Ultimately, *in vitro* and *in vivo* approaches should be combined for building novel strategies, with the aim to characterize the danger associated with the exposure to major groups of chemical contaminants. Over the last decades, novel questions in toxicology have emerged regarding chemicals which can interfere with the homeostasis of living systems. Many man-made molecules present in the environment and the food-web are suspected EDC, and there is a growing awareness of the public regarding the risks associated with such bioactive substances. Concerns about the possible adverse effects of EDC are based both on epidemiological evidences in human and on effects demonstrated in animal studies. In the EU, the REACH regulation entered into force in 2007, with the aim to gain a better understanding of the risks related to chemicals, and eliminate substances which raise high concerns. But the evaluation of EDC sets new challenges for toxicologists as well as health risk assessment agencies, as illustrated by ongoing controversies on the “low dose” effects of endocrine disruptors[2]. Interestingly, even for the best known model xeno-estrogens, the mechanisms of low-dose metabolic modulation is only poorly understood, and the observation of non-monotonic dose-response curves (*in vitro* and *in vivo*)[2,3] suggests a metabolic modulation based on multiple targets at the level of the cell, the tissue, or the whole organism. Since numerous proofs of evidence have demonstrated that chemicals could interfere with the endocrine system, the debate has gradually evolved towards a major question currently discussed by toxicologists: should such effects be considered as adverse effects or are they simply an adaptive response with no health consequences ?

## Materials and methods

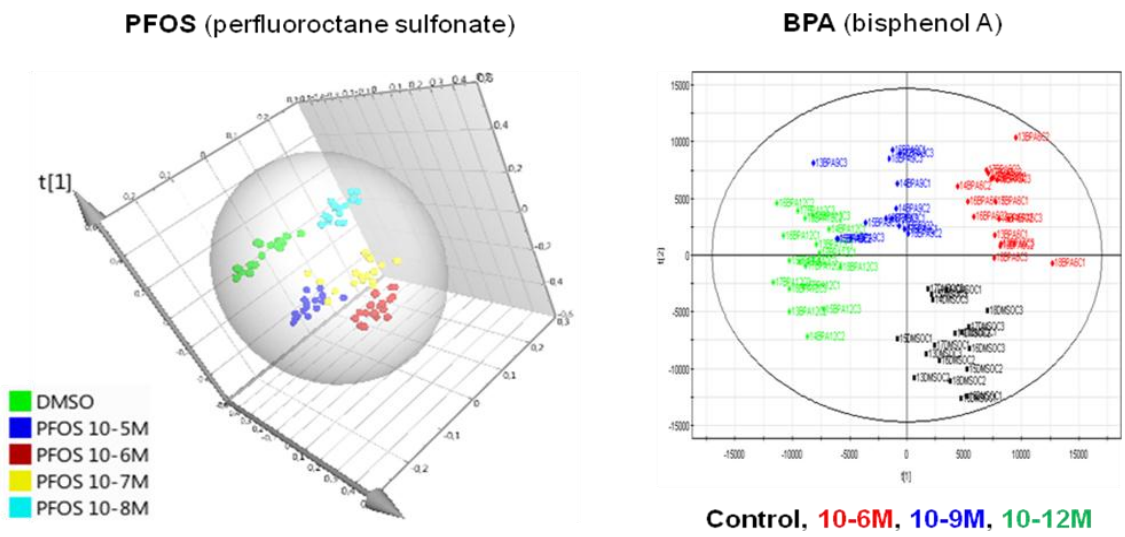
All samples used for the experimental studies illustrating the presentation were prepared for spectral analysis (NMR or MS) using protocols detailed previously (see Cabaton et al.[1]). Samples were submitted to $^{1}$H-NMR spectroscopy at 600.13MHz (Bruker Avance DRX-600 spectrometer fitted with a cryoprobe). For MS extracts, we used ultra high performance liquid chromatography coupled to high resolution MS (UHPLC-HRMS). The UHPLC system was a RSLC3000 (Dionex-Thermo Scientific, Les Ulis, France). Eluted compounds were detected using a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific les Ulis, France) equipped with an electrospray ionization source. For each spectrum, baseline and phase correction, data reduction, and bucketing multivariate analyses were used to evaluate the treatments on the metabolome. We first performed PCA to reveal intrinsic treatment-related clusters and detect eventual outliers. PLS-DA was then used to model the relationship between group and spectral data. We used orthogonal signal correction filtering[4] to remove variation not linked to the treatment. Filtered data were mean centered and scaled seven-fold cross-validation was used to determine the number of latent variables to be included in the PLS-DA models and to estimate the predictive ability ($Q^2$ score) of the adjusted models. The model is considered to be valid for a $Q^2$ score above 0.4[5]. Discriminant variables were determined using VIP (variable importance in the projection). We used this global measure of the influence of each variable on the PLS components to derive a subset of the most important metabolites for the separation of experimental groups. Then, we used the Kruskal–Wallis test to determine which metabolites were significantly different between groups. SIMCA-P software (V12; Umetrics AB, Umea, Sweden) was used to perform the multivariate analyses. Modeling of metabolic networks was carried out as detailed elsewhere[6] and with the help of the INRA Metexplore server we have developed (www.metexplore.fr).

## Results and discussion

**Novel systems biology tracks for the study of the low dose effects of chemicals.** Numerous chemicals are suspected to be responsible for biological effects at exposure levels way below the reference doses. Metabolic fingerprints, either based on nuclear magnetic resonnance (NMR) or mass spectrometric (MS) data, allow to unveil specific and significantly distinct metabolomic patterns, discriminating between exposed/non exposed groups, based on *in vivo* or *in vitro* experiments. A proof of concept was provided *in vivo* for animals according to their level of exposure to xeno-estrogens such as diethylstilbestrol (DES) or bisphenol A (BPA)[1]. Similar approaches can be successfully attempted in human beings[7]. But the human situation is more complex than that of laboratory animals, due to many factors including exposure conditions and a much higher genetic variability.

Moreover, biomarkers identified in biofluids are not the direct image of the metabolic impact on target tissues such as the liver, e.g. the main metabolizing organ. Hence, it is currently extremely dubious to link *in vivo* metabolic pathway modifications and observed metabolite concentration changes in biofluids. We expect that the interpretation of human metabolomic biomarkers will remain complicated until solid evidences can be obtained *in vitro* to support *in vivo* findings. Strong evidences were obtained using contaminants which raise concerns regarding human health, such as perfluorinated chemicals and bisphenols (figure 1 A & B).

Figure 1.A (left) and 1.B (right). LC-HRMS-based metabolomics discrimination of extracts of HepG2 cells incubated 24 hr with (A) different concentrations of perfluoroctane sulfonate (control: DMSO) or (B) different concentrations of bisphenol A (control: DMSO). Tri- (PFOS) and two- (BPA) dimensional PLS-DA scores plots. A (PFOS): 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ latent variables out of 4 components are displayed, with $R^2Y$=74.0% (22.4, 17.7, 19.1 & 14.8% on the 1$^{st}$, 2$^{nd}$, 3$^{rd}$ and 4$^{th}$ axis, respectively) and a $Q^2$ score of 0.619 (0.215; 0.215; 0.265; 0.158): model is valid and there are 76 VIP (Variables of Importance for the Projection) discriminating the different groups. B (BPA): 1$^{st}$ and 2$^{nd}$ latent variables out of 3 components are displayed, with $R^2Y$=89.9% and a $Q^2$ score of 0.661: model is valid and there are 23 VIP.




*In silico* analysis using the human metabolic network permits to decipher which processes are involved in the observed metabolomic shifts (Figures 2 & 3). This so called "genome scale model"[8] aims at gathering all the metabolic reactions the organism can perform into a single mathematical framework that can then be mined using dedicated bioinformatics algorithms[9]. Some of these methods are already available in the MetExplore web server developed by INRA (www.metexplore.fr).

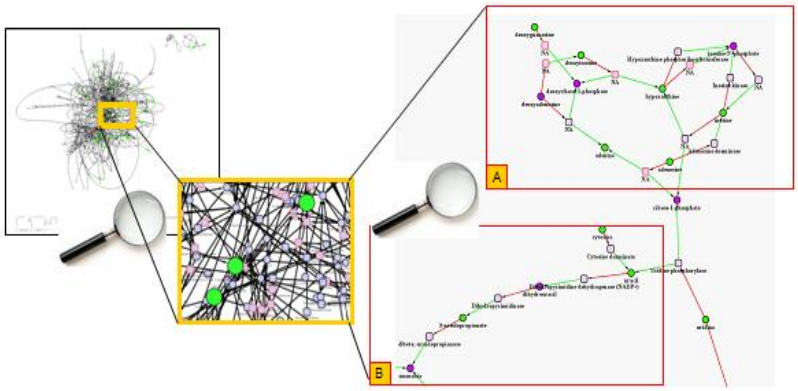



Figure 2. Network analysis of HepG2 metabolomics data in the context of the human metabolic network. From left to right: the entire metabolic network described in databases, a zoom view of the metabolic network (green dots are metabolites identified in the samples), and part of the metabolic network extracted by our algorithm connecting the identified metabolites in an interpretable "metabolic story" (A: purine pathway B: pyrimidine pathway).

**Conclusions:**

The development of global approaches in the field of toxicology, based on metabolomics and on the subsequent reconstruction of metabolic networks, opens new possibilities to explore the effects of candidate metabolic disrupters. Untargeted approaches of the metabolic shifts of human hepatic cell line models following chemical exposure, suggest that concentration-specific effects could be highlighted *in vitro*. For model estrogens, these metabolic shifts, in accordance with effects previously demonstrated *in vivo*[1,10], support the hypothesis of a modulation of the energetic metabolism. It is expected that specific shifts of the metabolome could be used in the future to predict potential effects of chemical contaminants, thus providing an interesting method for preliminary (or more advanced) toxicity screening. Despite whole body effects involving the disruption of homeostasis can by definition only be studied *in vivo*, the *in vitro* investigation of metabolic networks based on an untargeted

exploration of the metabolome through NMR and/or HRMS should provide useful hints about the toxicological profile and/or mechanisms of action of chemical contaminants, especially when low concentration of exposure are examined.

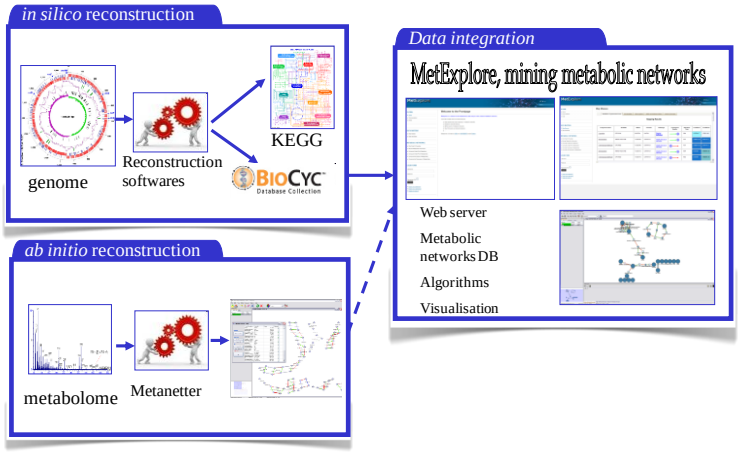



**Figure 3.** Reconstruction of the metabolic network based on the combined use of in silico databases and metabolomic data. The MetExplore server (www.metexplore.fr).

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